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FINAL REPORT

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Controlled Release of Antigens for One Dose Immunization

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ABSTRACT

Alum precipitation, vaccination regimen and controlled delivery by microencapsulation were studied to determine what criteria must be satisfied to provide a protective immune response to hepatitis B surface antigen (HBsAg) after a single injection of vaccine. In mouse studies, the 50% effective dose (ED_{50}) for the alum precipitated Heptavax B vaccine (Merck, Sharp and Dohme) was 3.8 ng when administered in a 3 injection regimen, but was 130 ng when one immunizing dose was used. Antigen release studies revealed that HBsAg is bound tightly to the alum, indicating that the antigen remains _in situ_ until scavenged by phagocytic cells. The ED_{50} with a 3 dose regimen of aqueous HBsAg was 180 ng, as opposed to over 2000 ng for daily injections of low doses for 90 days and 240 ng for a regimen that employed initially high doses that decreased geometrically at 3 day intervals over 90 days. The ED_{50} was 220 ng for a single dose regimen of HBsAg microencapsulated in poly (DL-lactide-co-glycolide) in a form that was too large to be phagocytized and had an antigen release profile similar to that achieved with the geometrically decreasing regimen of doses. This indicates that single injection of microencapsulated immunogens can achieve similar effects _in vivo_ to those achieved with multiple dose regimens. For HBsAg the effect to be achieved appears to be 3 pulses of particulate immunogens that can be scavenged by phagocytes.

INTRODUCTION

A major disadvantage of inactivated vaccines lies in their inability to confer lasting immunity. Due to rapid elimination from the body, multiple doses and boosters are usually required for continued protection[1]. Alum adjuvants, achieving their effects by mechanisms of antigen presentation and sustained antigen release[2], have been used successfully to increase the potency of several inactivated vaccines including those against tetanus, anthrax, and serum hepatitis[3,4]. Though useful, alum preparations are deficient in several aspects. Control over quantity and rate of antigen release is limited, often resulting in a continued requirement for immunization schedules consisting of multiple injections given over a period of several months to years. Alum adjuvants are also non-biodegradable and thus remain within the body, serving as a nidus for scar tissue formation[2] long after they have served their function.

Protracted, multiple immunization schedules are unacceptable during massive mobilization and deployment of troops. Changing global disease patterns and deployment of new biological warfare agents by enemy forces require flexibility in the number and types of vaccine antigens administered to soldiers departing for combat. Any immunization schedule requiring completion during engagement in non-linear combat would compromise this flexibility and place an unreasonable burden on our health care delivery system.

The main objective of this study was, therefore, to develop

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a biodegradable, controlled-release adjuvant system capable of eliminating the need for multistep vaccination schedules. This investigation was designed to: (1) determine in an animal model hepatitis B vaccine release rate characteristics desirable for single-step immunization, (2) incorporate those release rate characteristics into a one-step biodegradable poly(DL-lactide-co-glycolide)(DL-PLG) microencapsulated hepatitis B surface antigen (HBsAg) vaccine, and (3) conduct an in vivo trial comparing the effectiveness of this single-step vaccine against the conventional three-step hepatitis vaccine currently employed[5]. The results were intended to provide the foundation for further development of single step vaccines against hepatitis and other militarily significant diseases[6]. Keywords; Pharmacology, Antigens, Single Dose immunization, Medicine, Vaccine. Mice)

MATERIALS AND METHODS

Vaccine potency assay. Due to its availability, compatibility with cage mates, and potential application to the study of hepatitis B vaccine[7], the female Walter Reed (ICR) strain mouse was used. A hepatitis B vaccine potency assay for comparing the six-month immunization schedule currently in use[5] with that of a single-step immunization by sustained antigen release was established according to the following protocol: Specimens for baseline antibody titers were collected from twenty mice by exsanguination. Immediately prior to exsanguination, all mice employed in this and other exsanguination procedures in these studies were anesthetized with a 0.1 ml injection of V-

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Pento. Groups of 12 mice were then immunized according to a schedule consisting of either 0.25 ug, 0.025 ug, 2.5 ng, 0.25 ng, 2.5 pg, or 0.25 pg Heptavax B vaccine (HBV) administered in 50 microliter volumes subcutaneously (s.c.) at the beginning and end of the first, and end of the second month of the protocol. Antibody responses to the vaccine were monitored immediately before the third injection and approximately one month after the third injection. Specimens for antibody determination were collected by exsanguination of seven anesthetized mice from each group and assayed along with the baseline samples by the Abbott Ausab radioimmunoassay. Percent seroconversion verses micrograms vaccine employed was calculated by the method of Reed and Muench[8]. These data were employed to establish a mouse vaccine potency assay calibrated to detect differences between Heptavax B and other forms of hepatitis B vaccine.

In vitro antigen release rate from Heptavax B vaccine. Antigen release from aluminum hydroxide adjuvant in HBV was measured by pumping 2 cc per hour of 1:20,000 thimerosal in saline at $4^\circ C$ across a 0.2 u pore diameter Acrodisc filter apparatus containing 20 ug of vaccine. The effluent, collected by a Gilford fraction collector, was assayed periodically over several weeks for protein by UV absorption at 280 nm on a Beckman model 25 double beam spectrophotometer, and for HBsAg by the Abbott Ausria II radioimmunoassay made quantitative by using HBsAg standards supplied by Merk, Sharp, and Dohme. Accuracy of the HBsAg standards were verified by Biuret protein determination

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and by UV absorbance at 215 nm and 225 nm[9]. Nonspecific antigen retention on the Acrodisc filter was assessed by measuring percent recovery of a known quantity of HBsAg. Spontaneous degradation of vaccine antigen was monitored by comparing daily ratios of antigen to total protein detected in the effluent.

Evaluation of HBsAg stability. These studies were designed to characterize the stability of the aqueous antigen to the various physical conditions employed in the microencapsulation process. Conditions tested included lyophilization with reconstitution in distilled water, cyclohexane, methylene chloride, chloroform, methyl alcohol, acetone, iso-octane, hexane, acetone, pentane, or heptane; irradiation while lyophilized; and, exposure to elevated temperatures. Samples exposed to organic solvents were first lyophilized, reconstituted with the test solvent, evaporated to dryness under nitrogen at room temperature and reconstituted with distilled water. Test samples were compared against untreated controls by assaying serial dilutions of each with the Abbott Ausria II procedure and comparing the plots of counts per minute verses dilution.

Assessment of the effect of antigen release rate on vaccine potency. Three regimens simulating patterns of free HBsAg release that could be achieved by microencapsulation were contrasted with the three monthly dose regimen of Heptavax B for immunizing mice. To do so, 224 ICR mice were divided into groups and vaccinated as indicated below. Seven mice from each subgroup were exsanguinated at the end of the second and third months of

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the experiment. The sera were separated and assayed for specific antibody response to HBsAg by Abbot Ausab procedure.

HBV regimen a: 14 mice/treatment receiving 3 s.c. injections of 250, 25, 2.5 or 0.25 ng doses of HBV a month apart.

HBsAg regimen a: 14 mice/treatment receiving 3 s.c. injections of 250, 25, 2.5 or 0.25 ng doses of aqueous HBsAg a month apart.

HBsAg regimen b: 14 mice/treatment receiving total doses of 750, 75, 7.5 or 0.75 ng of aqueous HBsAg over 3 months by s.c. injections of ZX_y ng at 3 day intervals, where Z is the total dose, y is the injection number, and X is the fraction indicated on the graph in Fig. 1 minus the fraction for the previous injection.

HBsAg regimen c: 14 mice/treatment receiving daily s.c. injections of 8.33, 0.833, 0.0833 or 0.00833 ng of aqueous HBsAg for 3 months.
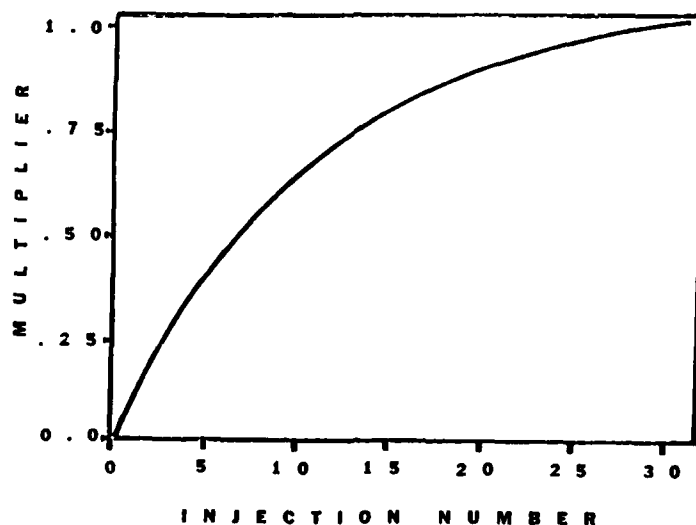

FIG. 1. Curve for determining vaccination dosages for regimen b.

Microencapsulation in DL:PLG. Microencapsulated immunogens were fabricated by Southern Research Institute, Birmingham, AL. DL-PLG polymers were synthesized from the cyclic diesters, DL lactide and glycolide, by using a ring-opening melt polymerization catalyzed by tetraphenyl tin[10]. The resulting polymer was dissolved in methylene chloride, filtered free of insoluble contaminants and precipitated in methanol. Lactide-co-glycolide mole ratio of the product was determined by nuclear magnetic resonance spectroscopy. Encapsulation of HBsAg in DL:PLG polymer was achieved by an organic phase separation process[11]. Microcapsules of the desired size (approximately 100 micron diameter in these studies) were isolated from each batch by wet sieving with hexane through standard mesh stainless steel sieves and then dried for 24 hours in a vacuum chamber maintained at room temperature.

In vitro analysis of encapsulated antigens. Integrity of encapsulated antigen was assessed by comparing the antigen to total protein ratios present in microcapsule hydrolysates with those obtained from suspensions of pure unencapsulated antigen. Centrifuge tubes containing 1 ug of either microencapsulated or pure vaccine antigen in 1 ml saline were incubated at 4°C with shaking. Samples were collected at weekly intervals by interrupting the incubation, sedimenting the contents of the tubes by centrifugation and withdrawing the supernates. Sediments were resuspended in 200 microliters of saline and supernates were assayed for HBsAg by the Abbott Ausria II

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radioimmunoassay. The HBsAg standard described earlier in this report was used as the calibrator. Antigen destruction due to the encapsulation procedure was monitored by a comparison between the antigen assayed from the hydrolysate and from the untreated antigen control.

Assessment of the potency of DL:PLG microencapsulated HBsAg for immunizing ICR mice when used alone and in combination with Heptavax B vaccine. HBsAg loaded microcapsules that had been fabricated by Southern Research Institute to release the majority of their HBsAg load within 40 to 50 days were serially diluted in 10-fold steps by mixing the dry, loaded capsules with blank placebo capsules of similar size and composition. The resulting stock and diluted microcapsule preparations were placed onto a lyophilizer when not in use in order to assure minimum spontaneous degradation prior to injection. On the day of injection, a predetermined weight of microcapsules or placebo-diluted microcapsules was added to each syringe. Immediately prior to injection either one or two ml of injection vehicle (2 wt % carboxymethyl cellulose and 1 wt % Tween 20 in water, Southern Research Institute) were drawn into the microcapsule-loaded syringes, mixed and injected. All mice were vaccinated s.c. as indicated below:

Group 1: 14 mice/treatment receiving 250, 25, 2.5, 0.25 or 0.025 ng HBV.

Group 2: 14 mice/treatment receiving 1000, 250, 25 or 2.5 ng aqueous HBsAg with Bovine Serum Albumin (BSA).

Group 3: 7 mice receiving 1600 ng microencapsulated HBsAg (mHBsAg) plus 0.25 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or 0.16 ng mHBsAg plus 0.25 ng HBV.

Group 4: 7 mice receiving 1600 ng mHBsAg plus 2.5 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or 0.16 ng mHBsAg plus 2.5 ng HBV.

Group 5: 7 mice receiving 1600 ng mHBsAg plus 25 ng HBV and 14 mice/treatment receiving 160, 16, 1.6 or 0.16 ng mHBsAg plus 25 ng HBV.

Group 6: 7 mice receiving 2500 ng mHBsAg and 14 mice/treatment receiving 250, 25, 2.5 or 0.25 ng mHBsAg. Fifty-three days after receiving the above injections, the mice were anesthetized with an 0.1 cc injection of V-Pento and exsanguinated. Blood samples were allowed to clot and the sera were separated by centrifugation. The serum samples were assayed for antibody to HBsAg by the Abbott Ausab procedure.

RESULTS

Heptavax B vaccine potency. As can be seen from Table 1, the total dose of vaccine which produced seroconversion in 50% of

TABLE 1. Potency of Heptavax B vaccine in ICR mice.

No. Inj.	ng Heptavax B per Injection							ED_{50} ng
	250	25	2.5	.25	.025	.0025	.00025	
2	5/5	4/4	3/6	2/6	0/5	1/4	0/4	1.7
3	6/6	6/6	4/6	1/6	0/6	1/6	1/6	2.0

* Number positive seroconversions per number vaccinated.

the vac_inated mice (ED_{50}) for HBV was approximately 2 ng, whether the vaccine was given in 2 or 3 injections.

In vitro antigen release rate from HBV. HBsAg release from the 20 ug of Heptavax was not detected in any of the 21 fractions of saline collected from the Acrodisc polycarbonate filter over a 30 day period. The lower limit of detection for the Abbott Ausria II assay employed was approximately 4.8 ng/ml. The Acrodisc filter used in the antigen release study was back-washed with 10 mls normal saline. Quantitation of the HBsAg present within this back-wash eluent revealed the presence of the original 40 ug of Heptavax vaccine which had been loaded into the filter at the start of the experiment. This is the concentration which one would expect to obtain if there had been no deterioration of the original 40 ug/ml HBsAg loaded onto the filter, none of the antigen eluted from the alum adjuvant, and none of the vaccine had adsorbed onto or passed through the filter.

Evaluation of antigen stability. Considerable effort was expended in assessing the effects of physical conditions on the antigenicity of HBsAg to insure that the conditions used for microencapsulation would not cause serious degradation of the immunogen. Since microencapsulation must be performed on dried materials which are suspended in organic solvents, the HBsAg, which was provided as a solution, had to be lyophilized. Initial attempts at lyophilizing HBsAg in normal saline resulted in a total loss of detectable antigen within samples. Dilution of the

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HBsAg sample 1:10 in distilled water prior to freezing resulted in preservation of nearly 100% of the antigen detectable in the original sample. Studies of antigen stability at elevated temperatures revealed that HBsAg may be heated to $50^{\circ}C$ for up to one hour without appreciable loss of antigen. The studies involving exposure of lyophilized antigen to organic solvents indicated that iso-octane and hexane had minimal effects on antigenicity, but that 95% to 100% of antigenicity was lost upon exposure to either methylene chloride, chloroform, cyclohexane, or methyl alcohol. Moderate antigen loss occurred in the presence of acetone, pentane and heptane. As a result of these studies, hexane was chosen as the solvent for microencapsulation.

Assessment of the effect of antigen release rate on vaccine potency. The results (Table 2) indicated that immunogen formulation (i.e., the alum adjuvant of Heptavax B) had far more

TABLE 2.   Effect of immunogen formulation and vaccination
regimen on potency for immunizing ICR mice.

Immunogen Formulation	Regimen	ng Total Dose HBsAg				ED_{50} ng
		750	75	7.5	.75	
Heptavax B	a	7/7*	6/7	5/7	1/7	3.8
Aqu. HBsAg	a	4/6	3/7	0/7	0/6	180
Aqu. HBsAg	b	6/7	0/7	1/7	0/7	240
Aqu. HBsAg	c	1/7	0/7	0/7	0/7	>2000

*   Number positive seroconversions per number vaccinated.
a   3 injections of 1/3 total dose a month apart.
b   Injections administered every three days for 90 days in decreasing dosages according to a logarithmic progression.
c   Injections of 1/90 total dose daily for 90 days.

effect on potency than did the vaccination regimen, and that pulsing with large doses of immunogen was more effective than

continuous administration of small doses.

HBsAg release from DL:PLG microcapsules. The microcapsules employed in this study were designed to disintegrate within three weeks after hydration. It is evident from the release curve (Fig. 2) that they performed as designed, releasing approximately 17% of their total load in an initial pulse and approximately 71% of the remaining available HBsAg over the first three weeks.
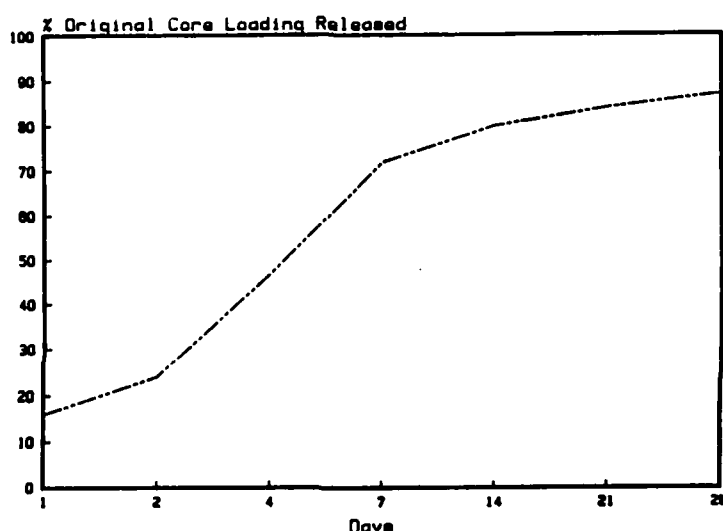

FIG. 2. Hepatitis B surface antigen release from 50:50 poly (DL-lactide-co-glycolide).

Assessment of the potency of DL:PLG microencapsulated HBsAg for immunizing ICR mice when used alone and in combination with Heptavax B vaccine. The results (Table 3) indicate that the microencapsulated HBsAg had approximately the same immunogenicity as did the Heptavax B. Neither immunogens were sufficiently potent to effect with a single injection seroconversion rates similar to those achieved after three injections of Heptavax B (Table 1). Only the immunogen

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TABLE 3. Potencies of Heptavax B and microencapsulated HBsAg by single injection S.C. when administered alone and in combination to immunize ICR mice.

Var. Dose Immunogen	ng Const. Dose mHBsAg	ng Variable Dose					Var. Dose ED_{50} ng	Tot. Dose ED_{50} ng
		2500	250	25	2.5	.25		
Heptavax B	0	13/14*	8/14	4/14	0/13		130	130
Heptavax B	0.16		11/13	4/14	1/14		1.7	1.8
Heptavax B	1.6		10/13	1/14	0/13		100	100
Heptavax B	16		3/14	1/14	1/14		>470	>490
Heptavax B	160		3/12	2/11	1/12		>370	>530
Heptavax B	1600		7/7	7/7	7/7		<0.8	1600
Mic. HBsAg	0	3/6	6/15	1/13	2/10	2/14	220	220

* Number positive seroconversions per number vaccinated.

combination of Heptavax B with 0.16 ng mHBsAg provided this level of seroconversion. At the ED^{50} endpoint, the 0.16 ng dose of mHBsAg is approximately 10% of the total dose. Similarly, a small amount of Heptavax B appeared to enhance the immunogenicity of the microencapsulated immunogen, although the combination was clearly less immunogenic when the two formulations were present at equivalent concentrations.

DISCUSSION

The potential advantage of microcapsules lies in their ability to be programmed during fabrication into forms that have quite different release profiles, including slow and steady release, multiple bursts of antigen over a period of time, or combinations of release forms. Sieving allows choice of microcapsule size, and the ability of DL-PLG to sequester antigen from the host's immune system until release occurs enhances control over exposure of the recipient's immune system to antigen

over a sustained period of time. These characteristics provided the impetus for these studies as they indicate potential for achieving the effects of a multiple injection regimen by controlling release _in_ _vivo_ after a single injection.

The results of these studies are important for gaining an understanding of the fundamental differences between the manner in which alum and microcapsules interact with the immune system. The antigen release studies showed that alum firmly bound the antigen on its surface, whereas the microcapsules sequestered the antigen load within the interstices of an immunologically inert polymer. Release of antigen from microcapsules was spontaneous and gradual while antigen release from alum was probably enzymatically mediated within host macrophages. Alum thus performed at least two useful functions as an adjuvant: by bearing its entire load of antigen upon its surface, it provided a large single exposure of antigen to the host; and, by being readily phagocytized by host macrophages, it served as a means of targeting the antigen to the immune system.

In order for microcapsules to be efficacious as a vaccine delivery system, a means of incorporating the two properties common to alum adjuvant must be devised. These properties, which were discussed above, are targeting antigen to the immune system and delivering the antigen load in a single concentrated pulse at its target. A gradual, sustained release of free antigen, as was achieved with the 100 micron microcapsules used in these studies, could be expected to elicit an immune response similar to that

seen with either regimen b or regimen c (Table 2), where multiple injections of small doses were employed. In fact, as shown in Table 3, the microencapsulated immunogen elicited a response similar to that achieved with regimen b. This is probably due to the fact that the microcapsules release approximately 10% of their antigenic load immediately after injection.

Microcapsules with extended release patterns tend to be large (>10 microns in diameter) and thus fail to be readily phagocytized. In order for the larger microcapsules with prolonged antigen release characteristics to be efficacious, the antigen eventually released from those microcapsules would have to be in a form which targeted and concentrated it within the recipient's immune system. This might be effectively achieved by microencapsulation of antigen coated alum or by microencapsulating clusters of smaller (<10 microns) microcapsules.

Microcapsules under 10 microns in diameter tend to be readily phagocytized and also tend to undergo rapid spontaneous degradation due to their high surface to volume ratio. These smaller microcapsules would be well suited for eliciting a primary response if their pulse of antigen release could be programmed to occur after phagocytosis.

REFERENCES

1. Spector, S.A. 1981. Immunoprophylaxis and immunotherapy, pp 770-793. In: Medical Microbiology and Infectious Diseases. A.I. Braude (editor), W.B Sauders Company, Philadelphia.

2. Jolles, P., and A. Paraf. 1973. Aluminum adjuvants in human sensitization. pp 106-108. In: Chemical basis if adjuvants, molecular biology, biochemistry, and biophysics, Volume 13. A.K. Kleinzeller, G.F Springer, and H.G. Willman (editors), Springer-Verlag, Berlin.

3. Brackman, P.S., and F.R. Fekety. 1958. Industrial anthrax. Ann. NY Acad. Sci. 70:575-584.

4. Maupas, P., A. Goudeau, P. Coursaget, J. Drucker, and P. Bagros. 1978. Hepatitis B vaccine efficacy in high risk settings, a two year study. Intervirol. 10:196-208.

5. Merck, Sharp, and Dohme. Heptavax-B Vaccine package insert.

6. Dean, J.A., and A.J. Ognibene. 1982. Hepatitis. pp 419-441. In: Medical Department, United States Army Internal Medicine in Vietnam, Vol II: General Medicine and Infectious Disease. A.J. Ognibene, O. Barrett (editors), Office of the Surgeon General and Center of Military History, Wash. D.C.

7. Gerety, R.J. 1979. News from the National Institute of Allergy and Infectious Diseases: Summary of an international workshop on Hepatitis B vaccines. J. Infect. Dis. 140:642-648.

8. Reed, L.J., Muench, H. 1939. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 27:493-497.

9. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the pricile of protein-dye binding. Anal. Biochem. 72:248-254.

10. Jackanicy, T.M, et al. 1983. Polylactic acid as a biodegradable carrier for contraceptive steroids. Contraception

8:227-234.

11. Kulkarni, R.K., E.G. Moore, A.F. Hegyeli, and F. Leonard. 1971. Biodegradable poly (lactic acid) polymers. J. Biomed. Mater. Res. 5:169-181.

14. Cutright D.E., P. Bienvenido, J. Beasley, III, W.T. Larson, and W.R. Posey. 1974. Degradation rates of polymers and copolymers of polylactic and polyglycolic acids. Oral Surg. 37:142-152.